Introduction

Streptococcus pyogenes [Lancefield group A Streptococcus (GAS)] is one of the most common and ubiquitous human pathogens. It is responsible for the majority of cases of sore throat in paediatric patients, but it is also the causative agent of severe life-threatening infections (sepsis, necrotizing fasciitis, toxic shock syndrome) and non-suppurative sequelae such as rheumatic fever and acute glomerulonephritis.1-5 In spite of more than 50 years of use of penicillin, S. pyogenes has not yet acquired resistance to this antibiotic and penicillin therefore remains the antibiotic of choice in the treatment of GAS infections. Erythromycin is an alternative antibiotic for the treatment of GAS infections in patients allergic to β-lactam antibiotics. However, a few years after the introduction of the antibiotic in 1952, Lowbury and Hurst6 reported the first erythromycin-resistant S. pyogenes isolate from a burns unit in a Birmingham hospital in the U.K. Subsequently, the isolation of erythromycin-resistant GAS strains was occasionally reported from various parts of the world, but generally remained unusual.7 The first reports of widespread and frequent erythromycin resistance in GAS came from Japan in the early 1970s.8-10 A positive association between erythromycin use and consequent increase in resistance was reported in Finland,11 and a reduction in the prescription of macrolides was associated with a fall in the resistance rates in Japan.12,13

Streptococcus pneumoniae is a common microorganism of the normal human respiratory flora. However, infection caused by the organism is one of the most frequent causes of pneumonia, meningitis, otitis media and bacteraemia in
children and adults worldwide.\textsuperscript{14, 15} Penicillin-resistant strains of \textit{S. pneumoniae}, first isolated in the late 1960s in Australia, are now prevalent throughout the world and are often associated with resistance to other antimicrobial agents.\textsuperscript{16, 17} Pneumococcal strains with intermediate and especially full resistance to penicillin G are often resistant to erythromycin and other 14-membered ring macrolides, such as clarithromycin and roxithromycin, and to azithromycin. In the USA, erythromycin resistance rates of 19–20\% were documented in isolates with an intermediate level of resistance to penicillin and of 49\% in penicillin-resistant isolates recovered from outpatients.\textsuperscript{18} In France, resistance to erythromycin increased to 29\% in 1990.\textsuperscript{19} Erythromycin resistance among streptococci can be due to target-site modification by an rRNA-methylating enzyme or by an efflux system. Target-site modification can be expressed either in a constitutive or inducible manner, resulting in co-resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS\textsubscript{B}). The \textit{erm} \textit{B} (erythromycin resistance methylase) gene class has been described in both \textit{S. pyogenes} and \textit{S. pneumoniae}\textsuperscript{21} isolates while recently \textit{ermTR} was described in \textit{S. pyogenes}.\textsuperscript{22} The macrolide efflux system, referred to as M-resistance\textsuperscript{23} or Novel resistance,\textsuperscript{24} confers resistance to 14- and 15-membered macrolides but not to 16-membered macrolides, lincosamides or analogues of streptogramin B. This type of resistance is characterized by the genes \textit{mefA} and \textit{mefE} in \textit{S. pyogenes} and \textit{S. pneumoniae}, respectively, which encode membrane-associated proteins.\textsuperscript{21, 25}

Sensitivity testing and antibiotic policy are dependent on the mechanisms of macrolide resistance in streptococci. Testing of erythromycin alone is no longer sufficient to assess the susceptibility of streptococci to all MLS\textsubscript{B} antibiotics. Therefore, in the present study, the in vitro activities of seven antibiotics were evaluated against recent isolates of \textit{S. pyogenes} and \textit{S. pneumoniae} in Belgium. The mechanisms involved were determined genotypically and phenotypically, and the clonal relationships between the macrolide-resistant strains were determined by \textit{M}-genotyping and pulsed-field gel electrophoresis (PFGE) in \textit{S. pyogenes}, and arbitrarily primed PCR typing in \textit{S. pneumoniae}.

### Materials and methods

#### Strains studied

A collection of 2014 Belgian clinical \textit{G. A. S} isolates (1607 isolated between 31 October 1993 and 1 November 1994, 203 from 1995, 103 from 1996 and 101 from 1997) were screened for erythromycin resistance by disc diffusion according to NCCLS criteria.\textsuperscript{26} The 1993 and 1994 isolates were collected during the course of a national research study on all \textit{G. A. S} infections (P. Descheemaeker, F. van Loock, M. Hauchecorne, P. Vandamme & H. Goossens, unpublished work), whereas in subsequent years strains mainly from invasive infections were submitted to our reference laboratory.

One hundred Belgian clinical \textit{S. pneumoniae} isolates were screened similarly for erythromycin resistance by disc diffusion. These strains were collected between 1995 and 1997 from several surveillance studies in Belgium.

### Susceptibility testing

Minimal inhibitory concentrations were determined by means of agar dilution according to NCCLS guidelines\textsuperscript{26} for the following antibiotics: erythromycin (Abbott Ottignies, Belgium), clarithromycin (Abbott), azithromycin (Pfizer, Groton, USA), miocamycin (Menarini Benefit, Zaventem, Belgium), rovamycin (Rhone-Poulenc Rorer, Vitry-Af Forville, France), clindamycin (Sigma Chemical Co., St Louis, MO, USA) and HM R 3647 (Hoechst Marion Roussel, Romainville, France), for the \textit{G. A. S} isolates, incubation was performed under aerobic conditions at 37°C for 24 h; for the \textit{S. pneumoniae} isolates incubation was performed under 5\% CO\textsubscript{2} at 37°C for 24 h.

The MIC resistance breakpoints were as follows: erythromycin, clarithromycin and clindamycin \(\geq 1\) mg/L,\textsuperscript{26} azithromycin \(\geq 2\) mg/L,\textsuperscript{26} miocamycin \(\geq 2\) mg/L.\textsuperscript{24} The resistance breakpoints for rovamycin and HMR 3647 were set at \(\geq 2\) mg/L.

#### Phenotypic detection of resistance mechanisms

Double disc test using erythromycin (diffusible content 78 \(\mu\)g) and clindamycin (diffusible content 25 \(\mu\)g) Neo-Sensitab discs (Rosco, Taastrup, Denmark) were placed 15–20 mm apart on a blood agar plate (Mueller-Hinton agar supplemented with 5\% defibrinated horse blood) on which a bacterial suspension equivalent to that of a 0.5 McFarland standard had been inoculated previously. Following overnight incubation at 37°C in aerobic conditions for \textit{G. A. S} isolates and under 5\% CO\textsubscript{2} for \textit{S. pneumoniae} isolates, the inhibition zone around the two discs determined the inducible, constitutive or \textit{M}-resistance phenotype of the strain.\textsuperscript{24}

#### PCR-based detection of the resistance genes

DNA was prepared using the rapid procedure described by Pitcher et al.\textsuperscript{27} The primers used to detect the genes \textit{mefA} and \textit{ermB} in \textit{S. pyogenes} and \textit{S. pneumoniae} were derived from Sutcliffe et al.\textsuperscript{21}

DNA amplification was performed with a DNA thermal cycler (model 9600 GeneAmp PCR system, Perkin-Elmer, Zaventem, Belgium). Each 50 \(\mu\)L PCR mixture contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 2.5 mM MgCl\textsubscript{2}, 0.1% Triton X-100, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 100 pmol of both primers, 0.5 U Pro-HA DNA polymerase (Eurogentec, Seraing, Belgium) and 100 ng genomic DNA. The primers used to detect the genes \textit{mefA} and \textit{ermB}, \textit{ermTR}, \textit{ermB}, \textit{ermTR}, \textit{ermB} and \textit{ermB} in \textit{S. pyogenes} and \textit{S. pneumoniae} were derived from Sutcliffe et al. and Sutcliffe et al.\textsuperscript{21}
50 ng of extracted DNA. All genes were detected using the same PCR running conditions, which consisted of an initial cycle of 3 min of denaturation at 93°C, followed by 35 cycles of 1 min of denaturation at 93°C, 1 min of annealing at 52°C and 1 min of elongation at 72°C, followed by one cycle consisting of 5 min of elongation at 72°C.

A flter amplification, the amplicon was mixed with 20 μL loading buffer (50% glycerol, bromophenol blue 0.8 mg/mL), 30 μL of which was electrophoresed in a 1.5% Pronase D1 gel (Sphaero Q, Burgos, Spain) for 1 h at 150 V in 0.5 × TBE (45 mM Tris–HCl, 45 mM boric acid, 1 mM EDTA) containing ethidium bromide 0.05 mg/mL. Visualization and image acquisition was carried out using the Gel Doc 1000 documentation system (Bio-Rad Laboratories, Nazareth, Belgium).

PFGE

Preparation of genomic DNA for PFGE analysis was done as described elsewhere. Briefly, cells from a culture that had been grown overnight on Todd Hewitt agar (Difco Laboratories, Detroit, MI, USA) were washed, adjusted to a density of 4 × 10^9 cfu/ml in EET buffer (100 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM Tris–HCl pH 8.0) and mixed with an equal volume of 1.6% (w/v) Low Melting Preparative Grade Agarose (Bio-Rad). The plugs were prepared in duplicate. A flter cell wall and protein digestion, one of the plugs was incubated overnight at 25°C in 250 μL of fresh restriction buffer containing 30 U SmaI (MBI Fermentas, St Leon-Rot, Germany) macro-restriction enzyme and the other plug was incubated overnight at 50°C in 150 μL of the appropriate buffer containing 30 U of SfiI mr enzyme. The electrophoresis conditions used for SmaI digests was ramping linearly from 5 to 35 s at a constant voltage of 6 V/cm over a period of 24 h at 14°C and for SfiI mr fragments the pulse times were ramping linearly from 30 to 90 s over a period of 24 h at 14°C and a constant voltage of 6 V/cm; both separations were performed in a 1% Pulsed Field Certified Agarose (Bio-Rad).

The DNA banding profiles were stained with ethidium bromide, visualized and digitized by the Gel Doc 1000 documentation system (Bio-Rad). Conversion, normalization and further analysis of the patterns was carried out using GelCompar software version 4.0 (Aplied Maths, Kortrijk, Belgium). The levels of similarity between the PFGE patterns were calculated by using the Dice coefficient and correlation coefficients were calculated by the unweighted pair group method with arithmetic averages.

Arbitrarily primed PCR typing

PCR-based typing was performed using the random primers D11344 (5′-AGT GAA TTC GCG GTG AGA TGC CA-3′), D14216 (5′-NNN AAC AGC TAT GAC CAT G-3′) and D8635 (5′-GAG CGG CCA AAG GGA GCA G AC C-3′) as described by Descheemaeker et al. The three arbitrarily primed PCR (AP-PCR) patterns obtained per strain were combined and compared with GelCompar version 4.0.

M-genotyping

The M-genotype of all S. pyogenes isolates was determined by hybridization of the denatured M-gene amplicon with M-type-specific probes in a reversed line blotting system according to the genotyping method described by Kaufhold et al. Twenty-six M-types were detectable with this method: M 1–M 6, M 8, M 9, M 11, M 12, M 13, M 18, M 19, M 22, M 24, M 26, M 29, M 33, M 41, M 46, M 49, M 52, M 53, M 57, M 60 and M 61.

Results

Susceptibility testing and phenotypic detection of resistance mechanisms

Among the 2014 S. pyogenes isolates, 165 strains demonstrated an erythromycin inhibition zone diameter of ≤20 mm and were further tested for their erythromycin MIC using the agar dilution method. Thirty-four strains showed MICs of <1 mg/L, while the remaining 131 strains (6.5%) were erythromycin resistant (MIC ≥ 1 mg/L): 94 from 1993–1994 (5.8%), 18 from 1995 (8.9%), 10 from 1996 (9.7%) and nine from 1997 (8.9%). Not a single isolate was inducibly resistant, 21 isolates were constitutively resistant (16.0%) and 110 strains (84.0%) showed the M-resistance phenotype.

Among the 100 clinical S. pneumoniae isolates, 33 demonstrated inhibition zone diameters of ≤20 mm and MIC ≥ 1 mg/L: 21 strains were constitutively resistant, nine were inducibly resistant, and only three strains (9.1%) showed the M-resistance phenotype.

The susceptibilities of the 131 erythromycin-resistant GAS and 33 erythromycin-resistant S. pneumoniae isolates to the seven antibiotics investigated are listed in the Table. All constitutively resistant strains, both GAS and S. pneumoniae isolates, were highly resistant to all antibiotics tested, except for HMR 3647, where the majority of the GAS strains (76.2%) and all S. pneumoniae strains were susceptible (≤2 mg/L). For the inducibly resistant S. pneumoniae strains, all were resistant to erythromycin, clarithromycin, azithromycin and rovamyacin, 33% and 78%, respectively, were resistant to miocamycin and clindamycin, and none were resistant to HMR 3647. The M-resistant GAS isolates were frequently resistant to erythromycin (100%), clarithromycin (91%) and azithromycin (86%), while they all remained susceptible to miocamycin, rovamyacin, HMR 3647 and clindamycin.
PCR-based detection of the resistance genes

The constitutive resistance phenotype and the inducible resistance phenotype were genotypically confirmed by the presence of the \( \textit{ermB} \) gene, while in all strains showing the \( \textit{M}-\) resistance phenotype the \( \textit{mefA/E} \) gene was present. Genes coding for both resistance mechanisms were never found in the same \( \textit{S. pyogenes} \) or \( \textit{S. pneumoniae} \) strain.

PFGE

All erythromycin-resistant \( \textit{GAS} \) were subjected to genomic analysis by PFGE. The inter- and intra-gel reproducibility of different restriction digests and electrophoretic runs was 100% as determined using the Dice coefficient (data not shown). All genomes were typeable by \( \textit{SfiI} \) analysis. For \( \textit{SmaI} \) analysis, only the constitutively resistant strains were typeable.
were typeable, whereas the majority of the strains with the M-resistance phenotype \((n = 92, 83.6\%)\) were non-typeable or resulted in a pattern that was difficult to interpret and was mostly characterized by the presence of a band of undigested genomic DNA (data not shown). Therefore, clonal relatedness based on Smal profiles was not studied further.

Visual and computerized analysis of all Sfi patterns obtained revealed 46 different patterns when a single band difference was used as a criterion to define a different pattern. Nine different patterns were detected among the 21 constitutively resistant strains, the largest cluster of identical patterns being composed of five strains isolated in the periods 1993–1994 and 1996. Among the 110 M-resistant strains, 35 different patterns were detected. One cluster of identical patterns, constituting 19.1\% \((n = 21)\) of the Belgian isolates, predominated and was composed of strains isolated in the periods 1993–1994 and 1995. Other clusters constituted less than 9.0\% of the investigated strains.

Arbitrarily primed PCR typing

Clonal relationships between all S. pneumoniae strains were investigated by computer-assisted comparison of the combined A P-PCR patterns. In total, 23 different patterns were detected among the 33 S. pneumoniae strains, of which 14 different patterns among the 21 constitutively resistant strains, six different patterns among the nine inducibly resistant strains and the A P-PCR patterns of the three M-resistant strains were all different.

M-genotyping

Among the 110 M-resistant GAS strains, M6 was the predominant M-type, detected in 29 strains. Other M-types detected among the M-resistant strains, ordered in sequence of frequency, included M2, M12, M4, M-non-typeable strains, M22, M1, M9, M11, M33, M60. The constitutively resistant strains included the M-types M6, M11, M22 and M-non-typeable strains.

In each cluster of identical PFGE patterns, an identical M-type was obtained. However, strains characterized by the same M-type regularly showed different PFGE patterns. The strains characterized by M6 showed seven different PFGE patterns. The cluster of indistinguishable strains predominating among the M-resistant strains was characterized by M6.

Discussion

Since the prevalence of the three erythromycin resistance phenotypes in S. pyogenes and S. pneumoniae in Belgium is presently unknown, the susceptibility of recent Belgian S. pyogenes and S. pneumoniae isolates to seven antibiotics was surveyed and the resistance mechanisms were determined phenotypically and genotypically.

Of 2014 Belgian GAS isolates, erythromycin resistance was detected by agar dilution in 6.5\% of the isolates. Although GAS isolates collected between 1995 and 1997 were predominantly from invasive infections as opposed to those collected in 1993 and 1994, we noticed an increase in erythromycin resistance of 5.8\% in 1993–1994 to 8.9\% in 1997. Thus, erythromycin resistance should be monitored with caution in Belgium. Indeed, erythromycin resistance rates have increased significantly in several countries such as Spain, Italy, Finland and Japan.

We found that the M-resistance phenotype was present in 84.0\% of the erythromycin-resistant GAS strains in Belgium. Similarly, this resistance phenotype became the predominant type in Europe, but data are lacking for the other continents.

The erythromycin-resistant GAS isolates in Belgium are polyclonal, as was shown by the different Sfi PFGE patterns and the different M-types detected among both constitutively resistant and M-resistant strains. Thus, the high prevalence of the M-resistance phenotype in Belgium cannot be explained by clonal expansion of a limited set of clones. This is in contrast to the predominance of a single T4M4 clone among the erythromycin-resistant GAS in Finland. In addition, the genetic determinants for the macrolide efflux system may be extrachromosomally located, since both M-resistant strains and several erythromycin-susceptible strains were detected, showing indistinguishable Sfi and Smal PFGE profiles (P. Descheemaeker, unpublished results). The genetic elements, being extrachromosomally located, may be transferred horizontally between different S. pyogenes isolates, which explains the polyclonal nature detected in the present study. The predominance of T4M4 in Finland was in part explained by the high erythromycin antibiotic selection pressure.

Remarkably, the majority of the M-resistant GAS isolates (92 of 110) were resistant towards Smal mr analysis while the constitutively resistant GAS isolates were typeable by Smal mr analysis. Resistance towards Smal digestion has already been observed by Cocuzza et al., who suggested that the genetic element encoding the M-resistance phenotype might also encode a Smal site-modifying activity that was repressed by the tet determinant.

Among the 100 clinical S. pneumoniae strains screened, 33 were erythromycin resistant (MIC \(\geq 1\) mg/L). Although this collection of strains was too limited to be conclusive on the prevalence of erythromycin resistance among S. pneumoniae isolates in Belgium, it is indicative of the prevalence of the different macrolide resistance mechanisms among Belgian S. pneumoniae isolates. In contrast to the GAS isolates, we found that only 9.1\% of the erythromycin-resistant S. pneumoniae isolates possessed the M phenotype. In the studies of Barry et al. and Johnston et al., the prevalence of the different macrolide resistance mechanisms among S. pneumoniae isolates was lower than in the Belgian isolates.
of the erythromycin-resistant S. pneumoniae isolates possessed the M phenotype. Clearly, the prevalence of the M phenotype among North American S. pneumoniae isolates is different from the situation in Europe. The predominance of the MLSB resistance phenotype among the Belgian S. pneumoniae strains was not biased by the presence of an outbreak strain, since 27 different A-P-PCR patterns were detected among the 30 MLSB-resistant strains.

No discordance was found between the resistance phenotypes, as detected by double disc diffusion, and the PCR detection of the ermB gene in the constitutively resistant strains and the mefA/E gene in the M-resistant GAS and S. pneumoniae strains.

As expected, the resistance phenotypes, and their respective resistance mechanisms, are also reflected in the antimicrobial susceptibility patterns. The M-resistant strains, encoding an efflux system, were mostly resistant to the 14- and 15-membered macrolides erythromycin, clarithromycin and azithromycin, but remained susceptible towards the 16-membered macrolides miocamycin and rovamyacin, the lincosamide clindamycin, and the ketolide HMR 3647. All constitutively resistant strains carrying the MLSB resistance mechanism were highly resistant towards all antimicrobial drugs tested, except for HMR 3647 (MIC < 4 mg/L). HMR 3647 has already been shown to be active against M-resistant S. pyogenes isolates and against S. pneumoniae isolates with high-level MLSB resistance. The mechanism of action of the ketolide is similar to that of the macrolides, but subtle differences in the site of action of these antibiotics may lead to a fundamentally different spectrum of activity. It remains to be proven clinically whether HMR 3647 can be used successfully for the treatment of S. pneumoniae infection with constitutive erythromycin resistance.

In conclusion, erythromycin resistance among GAS in Belgium remains relatively low and is mainly due to polyclonal S. pyogenes isolates showing the active efflux mechanism. Thus, miocamycin, clindamycin and HMR 3647 remain active against most erythromycin-resistant S. pyogenes strains. In contrast, erythromycin resistance among S. pneumoniae is high and the MLSB phenotype is the predominant mechanism. Here, miocamycin and clindamycin provide no alternative for erythromycin-resistant S. pneumoniae. Careful usage of macrolide antibiotics in therapy and continued surveillance of macrolide resistance in Belgium are warranted.

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